THE IDENTIFICATION OF TISSUES IN ARTIFICIAL CULTURES

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TEN FIGURES

In spite of the numerous studies upon tissue culture the usefulness of the method is still limited greatly by uncertainty in classification of many cell types most vigorous in their growth and of commonest occurrence. The nerve cell, the thyroid parenchyma, kidney tubules, ectoderm and a few other tissues are easily identified. But the ever varying linear, reticular and epitheloid growths are of undetermined origin. Although they are the predominating form in cultures they have only been referred provisionally to the group of embryonic sustentative tissues.

The structure of the growths is of only secondary importance in determining their classification. While it has by no means been established that tissues take on a more embryonic character in the plasma it is true that with few exceptions they assume the form of cell strings, reticula or membranes. Thus even the tubules of embryonic kidney are described by Lewis and Lewis ('12 b) as growing out in the form of a loose mesh. Much can be accomplished in the way of classification by comparing the growths from various organs and drawing inferences based on their tissue composition. In this way the probability has already been established that many of the common growths are derived either from supporting tissues or endothelium. Yet the character of the growths is too variable and too little under control to arrive at a final classification by indirect methods. They must be traced directly to their source in the parent tissue. For this purpose sectioned cultures are necessary. Little direct

evidence of this character has been obtained because up to the present time whole preparations have been used to the almost complete exclusion of sectioned cultures.

In the present study sectioned and whole preparations were used to supplement one another in identifying the growths. Cultures were made from chick ventricle of ages ranging from four to eighteen days. Limb buds of from four- to seven-day embryos were used. A much smaller number of series were made from liver and intestine. The comparison of the growths from the younger and older organs disclosed certain differences dependent upon the histogenetic stage of the organs. These are considered briefly.

The cultures were made in plasma according to the procedure of Carrel and Burrows ('11). The description of this method has been given too frequently to require repetition in detail. The plasma was taken from young chickens varying in age from two weeks to four months. Most vigorous growths took place in clots from a mixture of two parts of plasma to one of distilled water.

The preparation of cultures for sectioning presents considerable difficulty due to the marked tendency to shrink shown by the plasma clot and the very watery cells of the growths. Experience shows that care in adapting the technique of fixation and imbedding to the peculiarities of this material is especially worth while.

A brief description of the various types of heart ventricle growth will be given before considering the evidences as to their classification. The cultures from embryos of more than five days' incubation are divisible into a number of regions, four of which have a concentric arrangement determined by the rounded surface of contact between plasma and tissue. These are not well-defined in preparations of four- and five-day tissue because of the flowing and distortion consequent on the fluidity of the early embryonic tissue. Figure 1 shows them diagrammatically in a section of a culture cut parallel to the cover-slip. The central zone *a* is made up of tissue that remains for the larger part inactive. There is no evidence for the migration of any of

its cells outward although it is not possible to prove that some few do not leave the region. The boundary of the inactive zone does not become well-defined until degeneration has begun and the tissue external to it has become modified by the migration outward of its cells. Degeneration is often found in eighteen- to thirty-six-hour cultures to be confined to a central area much smaller than finally occupied by the inactive zone. Evidently death takes place first at the center of the tissue because of its remoteness from the plasma. The limits of the dead region then extend gradually toward the surface of the tissue. The inactive zone shows pyknosis and chromatolysis of the nuclei

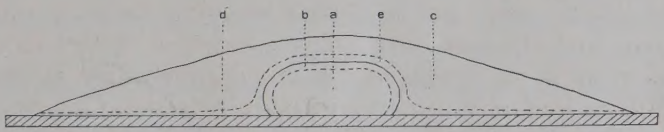

Fig. 1. Diagrammatic section of ventricle culture; *a*, inactive zone of implanted tissue; *b*, active zone of implanted tissue; *c*, region of reticular growth; *d*, cover-slip sheet; *e*, covering layer.

of heart-muscle cells. In two-week-old ventricle there is a clumping of the cytoplasm into large masses staining with basic as well as acid dyes. Epicardial and peritoneal endothelium have greater vitality than the heart-muscle. This also may be said for the endothelium of sinusoids except where the breaking down of the erythrocytes brings injury to the contiguous wall.

Surrounding the central zone except upon the cover-slip side, is a peripheral active region from which cell migration into the plasma takes place (fig. 1 b). It is never many cells thick and does not necessarily include all of the living tissue if the period of incubation of the culture has been short. In cultures of pulsating heart segments the cells contained in the active zone are put on the stretch at every systole. Fixation often causes the heart tissue to contract and thus preserve them in the condition of extension. Cell débris is usually present at the line of contact between tissue and plasma as a result of cutting the tissue from the parent mass.

Aside from a very fine, often degenerate reticulum, the remaining growths lying free in the plasma can best be described under two types, one fine and the other coarse. Neither of these give evidence of being separated by cell walls when stained with iron hematoxylin and erythrosin after Zenker or osmic acid fixations. There are all intermediate forms but many of the cultures are predominated by the one or the other type. Of the two kinds the finer is much more abundant. The nuclei in both varieties contain the one or two chromatic masses usually to be found in embryonic chick tissue. A measurement of the nuclei shows no constant difference in diameter from growths of heart-muscle, endothelium or reticular tissue. The cytoplasm of all cells in the plasma is watery and coagulates into a loose foam structure not resembling heart-muscle substance.

The finer mesh crosses the field at all angles. It differs from the coarse reticulum primarily in the much greater independence of its cells. It is made up of two intergrading cell forms of which one is elongated, slender and cylindrical while the other is polyhedral and usually triangular or quadrilateral in optical section. The ends of the first type and the angles of the other are drawn out into longer or shorter filaments. The cylinders may not be more than a micron in diameter although they are many micra in length. Their nuclei are forced to take on a rod shape by the limited diameter of the cells. The contact of the cells is at all times slight and often made only by the most delicate of filaments. The free ends at the periphery of the mesh send out fine pseudopodial processes. The two-cell forms intermingle freely in the reticulum.

The coarse mesh in all but the four-day cultures consists of bands 2 to 6 μ in width. They tend to flatten in the plane parallel to the cover-slip although they connect with each other at all levels. The growth has flowing outlines and forms loops which are characteristic in appearance and include spaces of more constant dimensions than found between the elements of the fine mesh. Nuclei are occasionally found side by side in the broader bands. The diameter of the narrower bands are no greater than usual for the fine mesh. In the nodes of the

coarser growth several nuclei occur all of which are in a plane parallel to the cover-slip. The triangular intersections of the strands 2 μ in diameter contain only one nucleus.

A sparse reticular growth of very fine texture often occurs in cultures of ventricle which have been injured in handling. By the use of dog plasma as a culture medium a similar growth is obtained. Both elongated and polyhedral cells are present in the fine mesh. The former are frequently drawn out into filaments not more than half a micron in diameter. The polyhedral cells also may be extended into such long threads at their angles that the central portion is much reduced in volume. The filaments are often tortuous and enlarged at successive points to give a bead-like appearance. The more normal part of this growth stains faintly. In other regions there is pyknosis and chromatolysis of the nuclei. The peculiarities of the growth are plainly an expression of decreased vitality and in many cases of actual cell death. In some instances it is the action of plasma from alien species that causes the injury. When the plasma is not responsible the growth comes from the tissues injured in cutting and handling. In this case toxic substances from the degenerating cells probably not only act upon the growth before it reaches the plasma but do harm by diffusing into the culture medium. Lambert ('12) describes a similar sparse and delicate growth from cultures of chick heart in rat plasma.

The cover-slip growth (fig. 1 d) is of almost as constant occurrence in vigorous cultures as the reticulum. It is so frequently in plain continuity with the latter that no doubt of the identity of the two is possible. Sections of many cultures made perpendicularly to the cover-slip show the reticulum grading into the cover-slip membrane and frequently stained whole mounts enable one to trace the mesh into the sheet growth. The continuity occurs most frequently close to the tissue where the growth is most crowded. It is often possible to make out a progressive flattening of the bands of the reticulum as they approach the cover-slip. Close to the tissue the cover-slip growth may be many cells in thickness. Tracing outward,

however, it soon flattens out into a single layer. The elements are often much flattened, especially at the border of the sheet and the nuclei may attain an area in this plane ten times as great as its usual cross section. The membrane growths agree with the reticular formation in the apparent absence of cell walls. While they have every appearance of a syncytial structure a final decision is impossible because a silver nitrate test for intercellular cement substance was not made. Aside from any differences as to cellular independence that may exist in various growths depending upon the greater or less development of cell walls it is certain that membranes show marked differences in this respect depending upon the extent of the spaces between the cells. The cover-slip growth associated with the finest mesh is made up of cells separated from each other by wide intervals of plasma and seldom intercommunicating by more than a few slender threads. The finer normal reticulum when flattened upon the cover-slip shows a frequent union by broad bands but intercellular communication may also be only by slender processes (fig. 6). In the membrane growth from the coarse reticulum broad attachments and multinuclear sheets occur.

The difference between the cover-slip growths corresponding to the coarse and fine mesh is especially noticeable at the border of the sheet. The former sends out cell bands which either project radially or form loops. The border of the membrane associated with the other mesh has a more finely broken border although its cells may also be connected with one another by their slender processes to form loops (fig. 6).

The third region of growth (fig. 1 e) of ventricle cultures is in the plasma next to the surface of the tissue. Burrows ('11) mentions its occurrence in early embryonic chick preparations but few others have referred to it. It is not confined to the earlier embyronic growths but is well developed from two weeks old ventricle. It has doubtless failed to attract attention because it can not be seen so well in whole preparations as in sections. It consists of flattened cells which are often piled upon one another to a great depth. Tangential sections from the surface of the implanted tissue opposite to the cover-slip

often show them to be as thin and sheet-like as at the border of cover-slip membranes. They are often elongated and rounded in cross section on the sides of the cultures. These dissimilarities of form are the result of the varying conditions of pressure in different regions of the culture brought about by the shrinkage of the plasma clot. The cells are in contact by slender filaments or less commonly by wide bands. Although frequently closely packed together a thin layer of plasma always separates them on most of their peripheries from neighboring cells. The transition of the layer into the reticulum and cover-slip sheet is usually gradual. As the reticulum is traced toward the tissue and into the covering sheet it is evident that even near to the tissue where the cells are the most flattened and in closest proximity they still form a compressed reticulum. Cell associations similar in make-up to the covering layer are often formed in relation to the free surface of the plasma or around droplets of serum contained within the clots.

The cultures from four-day ventricle give rise to an extremely heavy reticulum with bands often exceeding 15 μ in width (fig. 5). Their massiveness will be appreciated when it is recalled that the coarse mesh of older heart cultures are at the most 6 μ across. The appearance of the growth is also similar to the coarse mesh with its oval spaces. Thick masses apparently made up of the same tissues as the coarse mesh flatten out upon the cover-slip to form a sheet one cell in thickness at the periphery. Sometimes a finger-like form occurs in partial contact with the cover-slip and intermediate between membrane and reticulum. The great fluidity of both reticular and membrane growth is shown by their flowing outlines and lack of cell independence. A finer reticulum is also common in the four-day cultures which has nothing to distinguish it from the coarse form of the older tissue. The five-day cultures are intermediate in character between the growth from four-day and older heart. Figure 4 is from one of the finest reticular growths seen at this stage.

Before considering the evidence regarding the sources of the ventricle growth the tissues which compose it may be enumer-

ated. They include heart-muscle, nerve, endothelium and supporting tissue. Heart-muscle is, of course, the most abundant of these since it comprises the bulk of the myocardium. Nerve tissue is represented by a few neuroblasts. Endothelia include the endo- and peri-cardial layers and in the ventricle of the older embryos the walls of the sinusoids as well. The myocardium is separated on its inner and its outer surfaces from the endothelial covering by reticular layers. These consist of a mesh of formed substance upon whose strands are stretched cells which during embryonic development are becoming progressively more independent of their support. In the region of the atrial canal the sub-endocardial reticulum is continued into thickenings called endocardial cushions whose appearance is not unlike embryonic mesenchyme. Endothelium and reticulum approach the heart-muscle in abundance and like it are met on every cut surface.

In considering evidence for the growth of heart-muscle the cultures from six- to eighteen-day ventricle require consideration separate from that of four- and five-day tissue. Material from the former source gives no direct evidence for the participation of heart-muscle in the culture. Many hundreds of sections were searched for myofibrillae without success. Direct evidence for heart-muscle growth was obtained however in the sections of a culture from five-day heart which happened to include not only ventricle tissue but a portion of the atrial canal. The contents of the walls of the atrial canal was seen to be flowing out into a heavy band such as made up the coarser loop-like reticulum of the four-day ventricle cultures. The wall at this point is so thin and the process growing out from it relatively so large that the conclusion is unavoidable that the whole cell-mass was moving out into the growth. At six days muscle and reticulum make up about equal parts of the wall of the canal. At five days the tissue is in large part a primitive myocardial layer. Since there is every reason for supposing that the heart-muscle cells differentiate *in situ* the evidence is therefore very good that primitive heart-muscle cells take part in the growth. The appearance

of the coarse growths in unsectioned four-day cultures seems to justify their classification as primitive myocardium. Their strands broaden out in such a way at the base as to appear as a projection from the whole ventricle mass rather than from small portions of its surface. If primitive heart-muscle cells grow from five-day heart, it is, of course, probable that they are more frequently represented in growths of four-day ventricle. On the other hand, since the very heavy reticulum is rare in five-day cultures and entirely absent in growths of older tissue they probably do not grow from embryos more than five or six days old. These conclusions are in agreement with Burrows' ('11) observation of sparse growths of heart-muscle in a small part of his cultures of two-and-a-half-day chick heart.

A growth of nerve cells was not found in any ventricle culture. The neuraxones are so characteristic in appearance that they could hardly have escaped detection had they been present. Their failure to appear in the growth is doubtless to be explained by the smallness of their number and the consequent unlikelihood of their coming into contact with the plasma, for the responsiveness of neuroblasts to cultivation has been amply demonstrated by Harrison ('07), Burrows ('11), Lewis and Lewis ('12) and Ingebrigtsen ('13).

The abundant growths of the older embryonic ventricle and finer reticulum of the four-day organ which are not made up of heart-muscle tissue must evidently take their origin either from the endothelium of pericardium, endocardium or sinusoids if not from the reticulum. Few of the various studies of chick ventricle growths which have appeared are especially concerned with questions of identification. Lambert ('12) describes the mesh as of connective tissue origin and Burrows ('11) as mesenchyme. Lewis and Lewis ('12 b) think that it may be mesenchymal but consider the matter of its classification unsettled. In various growths of chick organs certain of these authors have suggested that flat polygonal cells may be of endothelial origin but have not traced their connection with the tissue. It is difficult to follow the growth either to endothelium or to reticulum. There

is confusion in the histological picture because of the cell débris which results from cutting the tissue. The collapse of the ventricular cavity and distortion of the tissue, especially in the younger ventricles, prevents contact with the plasma on surfaces sufficiently large to allow a proof of their continuity with the growth.

Cultures in which the pericardium and its reticulum are next to the plasma are better adapted for this purpose than sections through the endocardium covering the trabeculae of the spongy ventricle. Relatively large masses of pericardium may come in contact with the plasma while the endocardium is always intimately mingled with muscle. Figure 2 is from a photograph of a sectioned culture in which the active zone is made up of reticulum. The denser tissue internal to it is heart-muscle. Although the preparation was not killed until degenerative processes were well under way there is no difficulty in making out these tissues. The contrast between the two is seen much more clearly through the microscope than in the photograph because the muscle stains very intensely. A growth of fine mesh is plainly seen coming off from the reticulum. No traces of the pericardial endothelium which at first separated the reticulum from the plasma can be made out.

Figure 3 is from a photograph of a sectioned growth of the endocardial cushion of a thirteen-day heart. There has been no cutting with a knife. The free endocardial surface is in contact with the plasma. Because of the absence of the usual dead tissue resulting from cutting, strands from individual cells can be traced directly into the parent tissue. There is no room for doubt that the growth comes from the endocardial cushion. Just as the cushion is distinguishable from the reticulum by the presence of only one type of cell, so the elements of its growth are of a single form. They resemble the polyhedral cells of the fine reticulum.

There was no ventricle growth in which a connection could be traced with endothelium. The difficulties in the way of finding a region favorable for this purpose are too great to warrant concluding that endothelium does not take part. Indeed,

there is considerable reason to think that it does grow into the plasma. In the two cultures which have just been described the tissues concerned which are separated by endothelium from the plasma could not have grown into it had not the covering sheets ceased to bar their way. Inasmuch as no remnants of dead endothelial cells are to be seen, they have in all probability migrated into the plasma. Of the two types of ventricle reticulum the coarser has the greater similarity to endothelium. The finer mesh has already been shown to have its origin in part at least from sub-pericardial reticulum. It must not be forgotten in this connection that since the very fine degenerate mesh grades into the normal fine mesh and this again into the coarser variety, the claim is possible that since the first transition is due to differences of the plasma the change from the fine to the coarse type has a like explanation. Such an interpretation is not in agreement, however, with the conditions found in the heart or other cultures. The so-called normal fine mesh and the coarse variety are vigorous and abundant in growth. Their cells give every indication by structure and staining reactions of being healthy tissue. The very fine growth without question stands apart from these as a type modified by its struggle with an unfavorable environment. If the coarse mesh comes from endothelium and the fine mesh from reticulum the inter-mixture of the two may well result from their close association in the ventricle itself.

Lewis and Lewis describe two types of cover-slip membrane for chick ventricle cultures. One of these which they find to be syncytial is said to develop from nearly all embryonic chick organs. They think that it arises from mesenchyme or connective tissue. Their figures correspond closely with the cover-slip membranes associated with the fine mesh. They also occasionally get from heart a non-syncytial membrane with pigment deposits around the nuclei. This variety was not encountered in my preparations.

LIMB-BUDS

It is of special interest to study limb-bud and ventricle cultures together because the growth of pre-muscle cells can be compared with primitive heart-muscle. The degree of similarity of the mesenchymal growths from limb-bud and of ventricle reticulum also has significance in view of the uncertainty as to whether reticulum makes its origin from endothelium or directly from mesenchyme.

The five-day-old embryonic limb-bud consists of ectoderm and closely-packed mesenchyme in which the axial scleretogenous tissue is just beginning to differentiate as an especially dense region. In the surrounding zone the pre-muscle cells can be distinguished by their elongation parallel to the axis of the bud. The vascular system is represented by sinusoids. Nerve fibers have not yet extended into the bud. The ectoderm consists of a layer two cells in thickness. At seven days a difference can be made out in the form and staining qualities of the sub-dermal and the scleretogenous tissues. The pre-muscle cells are markedly elongated. Sinusoids now form an extensive system and nerve fibers have migrated in.

The difference in appearance of the cells of scleretogenous pre-muscle and ectodermal regions combined with their separation into independent zones render the limb-bud favorable material for tracing the growth to its parent tissue. The manner of growth of the limb tissue differed greatly from heart, due to the influence of ectoderm and mesenchyme. The former tissue invariably retains its continuity as a sheet and when it grows vigorously can limit the distribution of other tissues to the region internal to it. The mesenchyme often flows out *en masse* taking with it sinusoids and pre-muscle cells (fig. 8).

The ectoderm can easily be traced from tissue to growth either in sections or whole preparations. Its presence in the plasma is clearly in part due to a creeping of the edge of the sheet out into the plasma. The portion retaining its contact is often stretched into a thin sheet. In the plasma the ectoderm may form masses several cells in thickness. Single cells or

small groups occasionally project from the border of the sheet but there is always a considerable cellular contact.

The various mesodermal elements of the seven-day limb-bud are already sufficiently differentiated to give rise to a number of distinct growths.

Scleretogenous tissue can be identified in the plasma of many cultures close to regions of its contact with the plasma. Sometimes there is a migration of an entire region of the scleretogenous axis out into the plasma but the cells show no power of orientation and soon die. In most cultures the scleretogenous tissue is internal to mesenchyme and ectoderm and for this reason unable to reach the plasma quickly. Making due allowance for this handicap in position the vitality of the tissue from the seven-day embryo still appears to be of a low order.

The pre-muscle cells can be easily traced out into the plasma in many stained whole mounts because of their spindle form in the organ and their still greater elongation in the plasma (fig. 8). They form long strings which seldom branch. They are often long enough to reach the confines of the plasma drop and be deflected parallel to its surface. When in contact with the cover-slip the growth still maintains its elongated form thus differing from any other membrane growth (fig. 9). It is of interest that both primitive heart and skeletal muscle retain sufficient plasticity to appear in the plasma.

Another mesenchymal limb-bud growth is made up of large masses of cells formed, as in the case of the scleretogenous tissue, by the loosening up of the intercellular bonds of entire regions and a flowing out upon the cover slip (fig. 8). The cells are piled upon one another without a definite arrangement and at the outer border of the mass lie scattered upon the cover-slip in a much more irregular manner than in the membrane growths of heart reticulum. In figure 8 the growth is plainly seen to be intermingled with spindle shaped pre-muscle cells. This identifies it as the little differentiated mesenchyme surrounding the scleretogenous and the pre-muscle cells. From among mesenchymal cells of cut surface a reticulum often ex-

tends into the plasma which is apparently also mesenchymal in origin. The membrane associated with it is similar to the usual cover-slip sheet described with the fine mesh of the ventricle. The view that it is mesenchymal is strengthened by the fact that similar although somewhat more flowing and embryonic growths occupy the chief place in cultures from five-day embryos. If the reticulum as well as the more massive growth is from mesenchyme there is need of an explanation for the development of two such different types from the same tissue. A possible clue is to be found in the fact that the reticulum only occurs in connection with the free cut surfaces while mesenchymal cell-masses are associated with the cultures in which a scant plasma drop has flattened out the border of the implanted tissue by its shrinkage. Together with the flattening of these cultures there is always an extension of the ectodermal sheet out toward the periphery of the drop thus greatly limiting the plasma accessible to the mesenchyme and remaining tissues.

If it be correct to regard the reticular growth of the limb-bud as of mesenchymal origin, then heart reticulum and mesenchyme are closely similar in their growths and as far as this evidence goes are closely related. If, on the contrary, it should be shown later that mesenchyme gives rise only to the massive growth the arguments from tissue cultures would be in support of the origin of reticulum from endothelium.

LIVER AND INTESTINES

A few series of cultures from five- and eight-day intestine as well as from five- and ten-day liver were prepared as a basis for comparison of the growths.

A fine reticulum can be clearly traced in many sections to the mesenchyme of the intestine. Growths which arise from surfaces of the intestine with portions of the peritoneum intact often contain trabeculae of greater diameter than found where the growth is plainly of mesenchymal origin. It is not possible to trace the broader bands definitely to the peritoneum because the mesenchyme is always found to be taking part in the growth where the continuity of the peritoneum is lost. It is very prob-

able, however, that they arise from the peritoneum just as similar growths from heart ventricle also probably are of endothelial origin. Whole mounts of the intestine sometimes show a flattening down of a part of the organ, accompanied by the flowing out of the mesenchyme of the region upon the cover-slip just as seen in limb cultures. It is usually possible in these preparations to trace the peritoneum out as an unbroken sheet into the plasma. Lewis and Lewis ('12 b) describe and figure this type of growth and look upon it as peritoneal. The reticular growth which has just been described as present in sectioned cultures does not occur where there is such a flattening but is confined to free surfaces of the tissue.

Liver cells do not take part in growths from the ten-day organ. A coarse and a fine mesh occur with about equal frequency. Figure 7 shows the cover-slip growth associated with the fine mesh. The coarse form appears in many sections to come from the peritoneum. Elsewhere there is no proof of a deeper origin. Figure 10 showing the fine mesh growth is from a ten-day embryo and is fixed after twenty-four hours of incubation. No peritoneum is present in the culture. The growth can not come from connective tissue septa except possibly at a few restricted regions. In ten-day chick livers a reticulum is already present, as can be ascertained in part of the implanted tissue where degenerating liver cells have dropped out of a section. Endothelium is also present in large amounts in the form of sinusoids. The occurrence of mesenchyme in the embryonic liver has not been proven. It is therefore not possible to determine the source of the growth from the interior of the organ.

SUMMARY

Reticulum. The common reticulum with its corresponding membrane-growth is traceable to sub-pericardial reticulum in six-day ventricle.

Endothelium. There is indirect evidence for a coarse reticular growth from the peritoneum of liver (five- and ten-day) and intestine (five- and ten-day) and from the endocardium and

pericardium of ventricle (five- to fourteen-day). An unbroken sheet is found to arise from intestinal peritoneum under certain conditions (six-day).

Mesenchyme. Sectioned cultures of intestine (six-day) gave rise to a fine mesh similar to that of ventricle reticulum. Mesenchyme is sometimes given off from limb-buds (five- and ten-day) in the form of disorganized masses of cells. Under other conditions reticulum and a corresponding membrane growth apparently also take their origin from limb-bud mesenchyme.

Heart-muscle. In one sectioned five-day culture the contents of the wall of the atrial canal is found to be moving out into a strand of a very coarse reticular growth, such as is common in cultures from four-day ventricle. Primitive myocardium of four- and five-day heart therefore apparently grows out as a coarse mesh but it is unlikely that heart-muscle of older ventricle has this power.

Endocardial cushion of ventricle. A growth of polyhedral cells resembling those from sub-pericardial reticulum was traced in sections to the endocardial cushion (thirteen-day).

Scleretogenous tissue of limb-bud. The scleretogenous tissue can move out into the plasma for a short distance but has little vitality (seven-day).

Ectoderm of limb-bud. There is an extension of the ectoderm out into the plasma but this is due, in part at least, to a creeping of the original layer, as is shown by its marked thinning on the surface of the limb bud (five- and ten-day).

Pre-muscle tissue of limb-bud. The spindle-shaped pre-muscle cells of seven-day limb buds give a characteristic linear growth in the plasma and upon the cover-slip.

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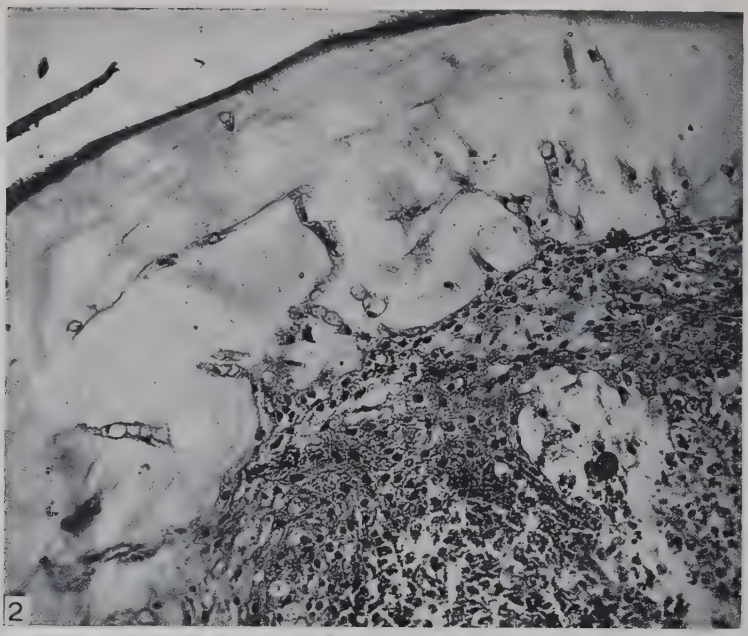

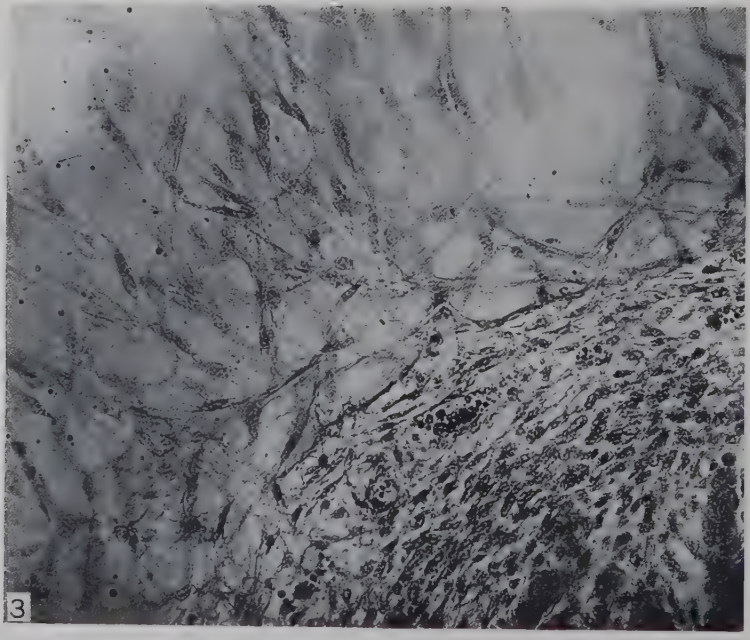

Fig. 2 Section of ten-day ventricle showing fine reticular growth arising from the reticulum. The more internally situated denser tissue is heart-muscle. × 600.

Fig. 3 Section of thirteen-day ventricle; the fine reticular growth is coming from an endocardial cushion. × 570.

Fig. 4. Fine reticulum of five-day heart; stained whole mounts. × 250.
Fig. 5. Coarse reticulum of four-day heart; stained whole mounts. × 270.

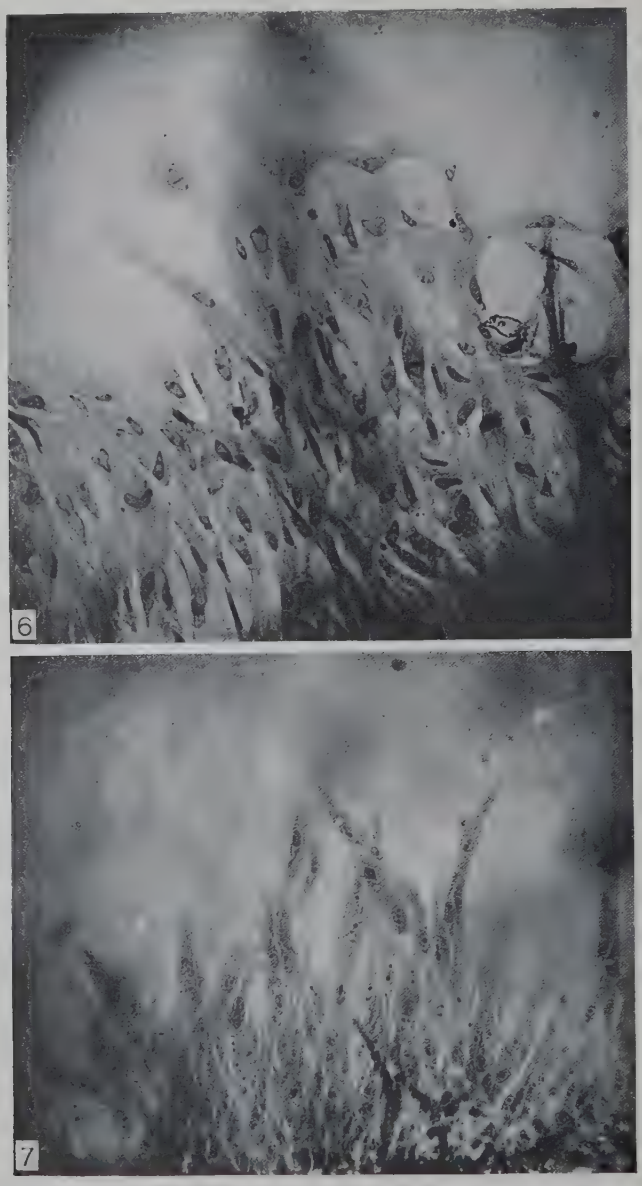

Fig. 6 Cover-slip growth associated with fine reticulum; nine-day ventricle; stained whole mounts. × 530.

Fig. 7 Cover-slip growth associated with fine mesh of five-day liver; stained whole mount. × 350.

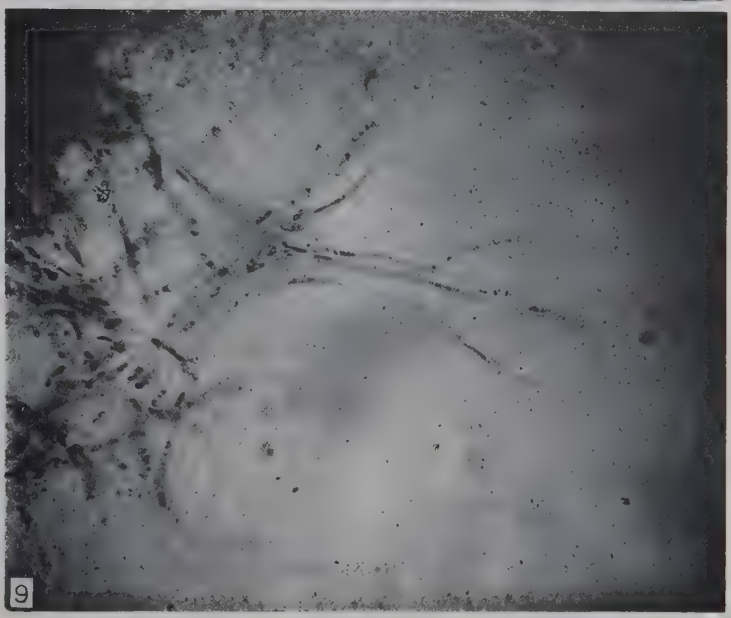

Fig. 8 Mesenchymal growth containing a few pre-muscle cells; spindle shaped pre-muscle cells are also seen projecting from the edge of the implanted tissue; stained whole mount. × 510.

Fig. 9 Cover-slip growth of pre-muscle cells from seven-day limb bud. × 410.

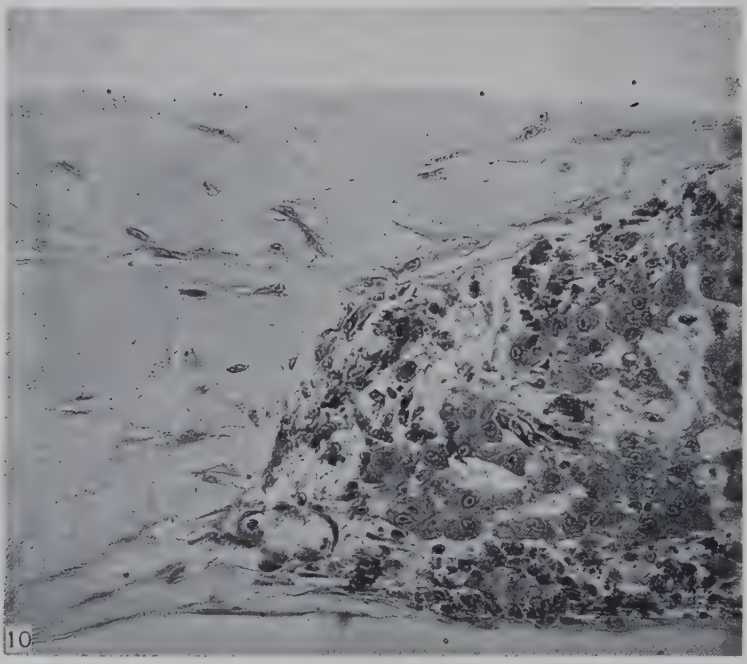

Fig. 10 Section of culture from ten-day liver, showing fine reticular growth. The cellular *débris* in the outer zone of the implanted tissue is made up of the remains of dead liver and sustentative cells. The inner zone shown at the right of the figure contains still uninjured liver cells. × 590.

THE ACTION OF ULTRA-VIOLET RAYS UPON THE FROG'S EGG

I. THE ARTIFICIAL PRODUCTION OF SPINA BIFIDA

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SIXTEEN FIGURES

While the method of the experimental embryological investigation detailed in the succeeding pages is of importance because of the constancy with which the condition of spina bifida may be produced in the embryo, the real value of the method and of its results lies in the interpretation which is thereby rendered possible of the physiological value of the several component portions of the fertilized ovum. The really fundamental problems to be determined by investigations of this nature are, first, whether the fertilized ovum is to be regarded as a composite structure made up of various system- or organ-anlagen, or the chemical progenitors or 'ferments' of such, distributed in definite and, perhaps, constant positions throughout the cytoplasm. Or, second, is the ovum to be considered in the sense of a unicellular organism, differing in no great respect from the physiological structure of unicellular organisms in general but possessing that specific potential to elaborate 'ferments' and pro-anlagen at successive genetic stages, and, ultimately the anlagen of the later developmental stages? In the former instance it is presumed that the embryonic parts are pre-localized in the cytoplasm of the ovum and make their appearance, in the words of Lankester, as "a sequel of a differentiation already established and not visible." In the second assumption the embryonic parts are unrepresented in the ovum, the regions of the cytoplasm being

then, so far as the future embryo is concerned, of equipotential value. It appeared to the author that a means by which a limited area of cytoplasm could be destroyed and yet left in its original relations to surrounding parts would afford a solution to this question. Accordingly, recourse was made to ultra-violet rays of such a degree of intensity as to cause the disorganization of the cytoplasm in from one to thirty seconds and of such a degree of concentration as to influence limited surface areas. Acting upon the suggestions made by Prof. E. H. Merritt, an apparatus was constructed which met these requirements fully.[1]

This apparatus consisted of a large induction coil actuated by a 110-volt direct current reduced by an unknown resistance. The potential, moreover, was raised by means of several Leyden jars shunted between the electrode wires. The terminals were made of iron, and were spaced about 5.0 mm. The eggs used for the purpose were those of the various forms of frogs occurring in the neighborhood of Ithaca, New York. These were obtained early in the morning, as soon after laying as was possible. At the time at which they were influenced they were in the undivided stage. Development was allowed to progress in the laboratory in some instances, and the eggs influenced at several later developmental stages, but no egg further along in its cycle than about the 64-cell stage was used. Furthermore, care was taken to reject such eggs as were collected late in the laying season for that particular species of frog, and particularly those located near the center of the egg bunches, specifically to avoid dealing with those possessing a tendency towards abnormal development. In preparation for exposure to the rays, the eggs were freed from their jelly, which had been found impervious to the light, and placed under a perforated tinfoil diaphragm. The perforations differed in size in different experiments. After the egg had been rotated so that a predetermined part had been brought directly under the center of a circular perforation in the dia-

[1] At this point I desire to express my indebtedness to Professor Merritt for helpful suggestions and to the Department of Physics of the University for the use of the apparatus with which the experiments mentioned in this paper were conducted.

phragm, both were then brought under the electrodes of the apparatus and the circuit closed.

While in the numerous experiments conducted, the various portions of the white and of the black hemisphere and of the equator of the frog's eggs were influenced in order, the author decided to limit the scope of this present communication to those effects produced by the rays when influencing the white hemisphere and the equator of the egg. Indeed, in addition to the significance of the findings of the investigation in the interpretation of the larger problem of ovum structure, the immediate purpose in presenting this paper is to establish the fact that the condition of spina bifida may be produced at will by this method.

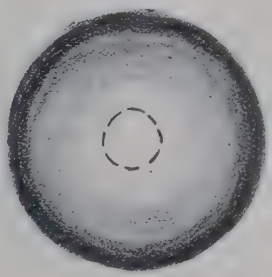


Figure 1

The aperture in the diaphragm used for this experiment was 0.4 mm. in diameter. The eggs averaged 1.7 mm. in diameter. Consequently but a small surface area proportionately of the total area of the egg was influenced. The latter amounted to 425.0 sq. mm. whereas but about 5.0 sq. mm. of this surface could be influenced by the rays. The relative sizes of these areas is brought out more clearly by reference to figure 1, which represents by a broken-line circle the portion of the surface area of the egg sphere illuminated. Further, it was found that the depth of penetration of the 0.4 mm. pencil of rays depended upon the length of exposure to the light. Uniform exposures of 30

seconds were employed in this series. A section through an egg so influenced is shown in figure 2, in which the depth to which the rays had penetrated is represented by the shaded portion on the right of the sketch. In this instance the rays passed in the plane of the section and at right angles to a tangent at the center of the surface of the affected area. The direct results of the illumination were corroborative of those previously observed by other investigators using violet rays, such as granulation of the chromatin and certain degenerative changes noted in the cytoplasm. No attempt was made, however, to study this aspect of the influence of the rays. It was noted in extremely long exposures of from 1 to 10 minutes, that masses of protoplasm

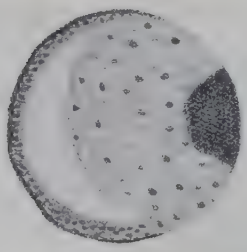


Figure 2

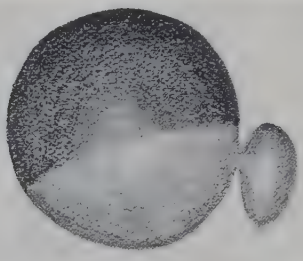


Figure 3

were in some instances extruded upon the egg surface, retaining, however, a slender connection with the main mass of the egg. In instances of such exovation, the egg died early after having made but little developmental progress. Such an exovate is shown in figure 3. It is of importance to note the fact demonstrated by the sketch that the mass of the exovate was approximately equal to that of the influenced area of the egg (compare with figure 2). The most plausible inference to be drawn from this phenomenon, in the terms of the interpretation of the ovum as an organism, seems to be that of an effort on the part of the ovum to rid itself of the chemically altered or dead protoplasm which can only act as a hindrance to its further developmental progress.

Reference to various series of experiments selected at random bring out the value of the method in the constancy of production of the condition of spina bifida. In one series of thirty-one 16-cell eggs used at the beginning of the experiments and exposed to the 0.4 mm. ray for 30 seconds each—various regions of the equator and of the white hemisphere being influenced—twenty-one developed abnormally, and but ten normally. Of the abnormal embryos, eight presented the condition of spina bifida. In another and later series of fifteen eggs in the 4-cell stage, influenced in the same manner, none developed normally. Most of these died during the early stages. Four, however, lived to swimming forms with two tails. Later in the spring, after the technique had been still further perfected and the eggs of the green frog were available, from which it was possible to remove the enveloping jelly more readily and more completely, the percentage of spina bifida embryos rose. In one set of five undivided eggs influenced in a similar manner, one died about twelve hours after the experiment, having made no developmental progress, and the four others grew to swimming forms presenting the condition of spina bifida, each having two tails. This last instance is merely representative of the high percentage of these forms of malformation obtained when the white hemisphere is influenced by the ultra-violet rays.

As has been mentioned above, the most effectual barrier to the penetration of the rays was the investing jelly. When this was completely removed an exposure of 10 seconds was sufficient to influence the egg. The presence of a very thin layer, however, completely blocked the passage of the rays even during an exposure of as much as 10 minutes. The author attributes most, if not all, of the irregularities in percentage production of spina bifida embryos to the presence of this jelly. The later results of the experiments were sufficiently assuring to warrant the conclusion that, when it could be positively known that the rays under the above conditions had actually penetrated the ovum in the regions above mentioned, the condition of spina bifida could be invariably brought about in the developing embryo. Taking these difficulties into consideration, however, the per-

centage production of the condition ranged between 85 and 90 per cent of the total number of eggs used.

An observation that recurred repeatedly was to the effect that the developmental period required by the eggs was lengthened as a result of the rays' influence. Under laboratory conditions ordinarily from 3 to 4 days were sufficient for the appearance

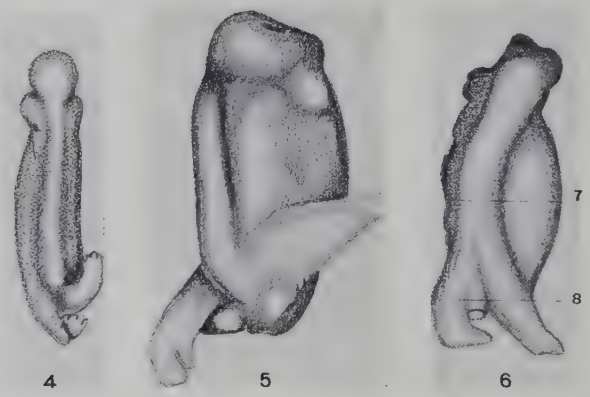


Fig. 4 A specimen of cauda bifida demonstrating the asymmetry of the tails. In this egg the rays had struck a portion of yolk farthest removed from the equator. The tails are provided, as is shown, with peculiar toe-like processes on their free extremities.

Fig. 5 In this tadpole the right tail encountered the body axis at an acute angle directed anteriorly. Two yolk plugs are to be seen, and the same splitting of the extremity of the left tail as was noted in figure 4.

Fig. 6 The lines 7 and 8 on this cauda bifida tadpole indicate the level of the cross-sections shown in the respective figures. In figure 7 the notochord lies ventral to the well differentiated neural tube. In figure 8 the asymmetry of the halved neural tube is shown, more particularly on the right side. Ventral to this lies the notochord, all traces of which are absent from the left half of the sketch. This right neural tube-half lay in the more actively used tail.

of the free-swimming tadpole-forms of the green frog; in the case of the experimented eggs, however, 5 or 6 days were required and in some instances 8 days. Furthermore, it was noted that during the 12 hours immediately ensuing upon the experiment the eggs seemed to have entered into a condition of temporary suspension of development, later resuming that process but with greatly lengthened tempo.

The further observation was made that free-swimming forms, such as are represented in figures 4, 5, and 6, seemed to be able to move about by the use of either tail, but that the swimming movements were more vigorous in one than in the other. This is an interesting fact in connection with the results obtained by study of the microscopical sections of the same specimens. In these it was learned that in the more favored of the two tails the neural tube was greater in diameter and extended a longer distance towards the tip of the tail. In some of the specimens,

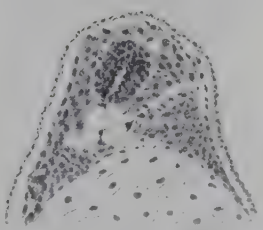


Figure 7

Figure 8

as is to be seen in figure 8, the notochord was limited to one tail. Figures 7 and 8 are cross-sections of the tadpole represented by figure 6. The right was the more active of the two tails during life. In figure 7 the neural tube, cut just cephalic to its bifurcation, is seen to be well differentiated, with the notochord lying ventral and adjacent to it. The section of figure 8 was taken immediately caudal to the bifurcation and shows the notochord confined to one (the right) tail.

Several specimens presented a peculiar relation of one tail to the longitudinal axis of the body; such are figured in 5 and 9. In the latter figure the main axis of one tail joined that of the trunk at almost right angles, whereas the other tail coincided fairly well with the main body axis. In figure 5 the right tail

met the body axis at an acute angle, looking forward. Such tails were, of course, useless from the functional standpoint; but their importance cannot be overestimated in furnishing exaggerated examples of the asymmetry of some of the types of spina bifida, such as were observed above in the cross-sections.

The study of the serial cross-sections demonstrated, furthermore, as these were followed in order caudally, that just posterior to the level of bifurcation of the neural tube each half tube des-

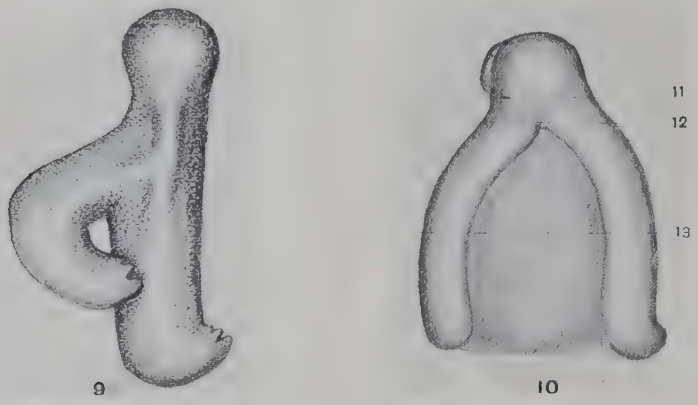
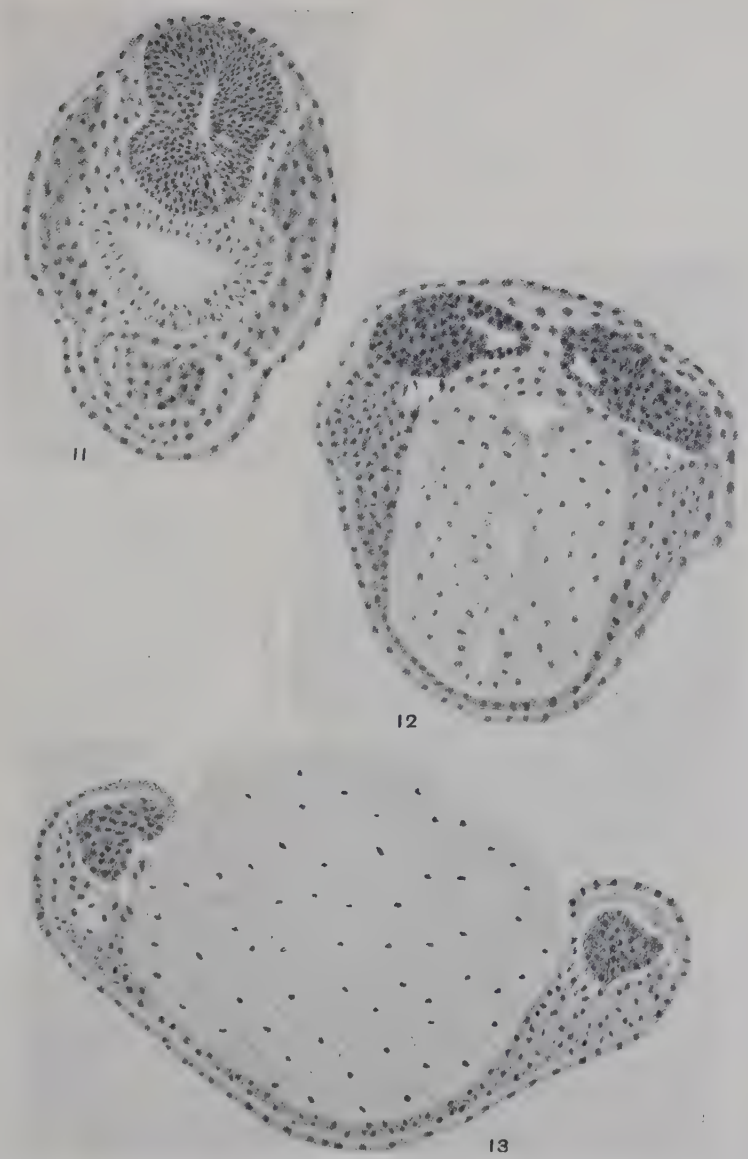


Fig. 9 This figure illustrates a marked instance of asymmetry with a division of the cord well anterior on the embryo. Here again the extremity of each tail is broken up into toe-like processes.

Fig. 10 In this embryo the rays had encountered an area well up on the equator in the median plane; hence, the bifurcation of the neural cord immediately posterior to the optic anlagen. The lines 11, 12 and 13 indicate the levels of the respective cross-sections illustrated by the succeeding figures.

tined for each tail presented an asymmetrical outline, the lateral wall being considerably thicker than the median. This is shown in part by the right neural tube in figure 8. As the series was followed farther caudally, however, a readjustment of the tube cells was observable, each half now becoming either a solid rod or a tube entirely symmetrical so far as the thickness of its walls was concerned; (see also figures 12 and 13).

The neural tube was caused to bifurcate at various levels, dependent upon the portion of the hemisphere influenced.



Figs. 11, 12, 13 In figure 12 the marked asymmetry of the neural tube halves immediately posterior to the level of bifurcation of the cord is shown. The neuroblasts become adjusted farther posteriorly, however (fig. 13) to form a more symmetrical tube or column of cells.

Where the rays struck close to or on the equator in the median plane of the egg the subsequent bifurcation was noted well forward towards the head region; figure 10 well demonstrates this fact. In figure 9 the point encountered was much lower down on the hemisphere, while in figures 6 and 4 it was still farther posterior. Such are really instances of cauda bifida. Another fact of the utmost significance was gained from studies of the histological sections. Since it was definitely ascertained that the rays had killed the portion of the egg illuminated, in no specimen was there to be observed, however, a deficiency or falling out of a portion of the neural tube, as one would expect

if the proanlagen or anlagen had been encountered by the rays. Notwithstanding the possibility of post-generation, with a replacement of the anlagen thus rendered inactive, the conclusion might be justified, tentatively at least, that the proanlagen are not located in the early stages of division of the ovum either in the yolk hemisphere or along the equator, but are confined wholly to a region well up on the black hemisphere. Before referring in detail to the results of other investigations dealing with spina bifida it will be well to emphasize some general considerations which must be taken into account in connection with the production of abnormalities.

It is taken for granted that there are many linking factors associating the developmental processes concerned in the pro-

duction of spina bifida with those of other forms of malformation occurring in nature and produced experimentally. Accordingly, one cannot well study the one without taking into consideration those general fundamental physico-chemical factors of development underlying both, upon the disturbance of which the production of anomalous conditions is dependent. The fact is well supported that the processes of differentiation in the tadpole, as in some other forms, appears to be dependent upon a series of complex and progressive chemical reactions which are of the nature of oxidations. In the later stages of development we are dealing with an organism whose chemical constitution differs considerably if not completely from that of the undivided ovum. The results of such reactions and chemical changes become apparent in the differentiation of the various anlagen. Eggs placed in pure water free from chemical compounds which might enter into the chemical structure of the organism, but supplied with a liberal amount of oxygen, can and do undergo the several processes of differentiation, finally hatching and swimming about. From that time on, however, when growth of the differentiated parts is initiated the organism requires a supply of various chemical substances for its appropriation. In this connection, it is significant that the great majority of defects produced experimentally are referable to changes in the differentiative stages of the embryo and only secondarily to defective growth phenomena.

Bearing in mind, then, the chemical modifications occurring during the differentiating cycle of the embryo, it is fair to assume that at certain specific times in the genesis of the anlagen their chemical composition is such as to render them more ready participants in chemical reaction with the chemical agent employed. Results so obtained cannot be cited without reservation as applying to the state of the undivided ovum, consequently much of the chemical work conducted along this line is subject to considerable qualification and limited in the insight which it furnishes into the constitution, chemical or physiological, of the ovum. Before the full truth can be established on this point, it must be demonstrated that the toxic action of the chemicals employed was exerted upon the egg during the undivided state

and not afterwards; or, in other words, upon the chemical 'ferments,' or proanlagen, and not upon the anlagen cells when they have attained what might be termed a condition of chemical completeness. Only by this means can we decide between the two conceptions of the ovum; as an organism elaborating its organ anlagen at succeeding developmental periods, or as a composite, mosaic-like structure.

While the artificial production of defects has been known for a long while to be possible, but very few have succeeded in advancing any plausible explanations covering the instance of spina bifida embryos. There are two aspects to the problem of the artificial production of spina bifida which are brought out in a review of the literature dealing with this subject. The first consideration is whether we are dealing with the action of an external agent upon some specific substance in the egg; and the second, whether the nature of this reaction is specific, referable to the agent alone, which possibly reacts upon the egg as a whole.

Morgan in 1894 and O. Hertwig in the year following were both successful in the production by chemical means of a large percentage of embryos showing the defect of spina bifida. That 0.625 per cent solutions of sodium chloride should produce so high as 50 per cent of this form of malformation was an argument in favor of a definite specific chemical or physical property of the compound. Prior to this date observers had recorded only occasional instances of this defect and had failed to give a convincing indication of the nature of the upset in the physico-chemical factors concerned. Roux was the first to call attention to the occurrence of spina bifida among frog's eggs, owing, apparently, to conditions found in nature. Panum recorded 38 instances of spina bifida in chicks, among 404 monsters produced. He, with Dareste and Féré, obtained monsters of various kinds by the employment of variations in temperature (as did Hertwig), by varnishing the egg shells, by shifting the long axis of the egg to the vertical, by traumatic injuries, shaking, magnetism, electrical means, various gases, vapors of lavender and by injecting different toxines and chemicals, such as turpentine, an-

iseed, absinthe, and cloves into the white of the egg. Their inability to associate any given deformity with a known and controllable cause led to a failure in the analysis of the normal developmental factors of the embryo. Richter found three instances of spina bifida among several hundred hen's eggs upon which he had experimented. Spemann, however, produced two-tailed embryos by simply tying a ligature between the two blastomeres, demonstrating the bilaterality of the anlagen but throwing no light on the nature or antero-posterior extent of the organ-building substances. Fol, Rauber, Born, and O. Hertwig attributed the duplicity to double fertilization. This explanation was too compromising regarding the anterior portion of the embryo, and later was found to be unnecessary. Godlewski's experiments with reduced pressure, and Herbst's with lithium salts, Morgan's with the centrifuge, Samossa's with atmospheres of nitrogen and of hydrogen, and Wilson's with Ringer's solution and with sodium chloride, furnish additional evidence of the diversity of ways by which this abnormal condition may be produced.

In this connection, it is interesting to note that Mall has reported 12 instances of spina bifida among 163 pathological human embryos, attributing as a possible cause of the condition, faulty implantation of the embryo. Analysed still further, however, by analogy to the conditions found among lower vertebrates, it seems possible that the human ovum, too, requires but little else than a good supply of oxygen for its differentiation during the early stages of development. At this time the causal forces are operative for the production of spina bifida. Undoubtedly, a deficiency in the supply of oxygen could be brought about by the imperfect imbedding of the ovum in the uterine mucosa. Bearing this fact in mind in connection with the features of differentiation of the ovum given on page 375, it seems superfluous to seek an explanation of the condition in man through the action of chemical substances or of altered temperature. Though the possibility of the direct or indirect dependence of the processes of oxidation upon the action of the latter agents must be

admitted, reasoning from conditions as we find them in the frog, a sufficient and probably a more primal cause, at least, is referable to faulty oxidation.

Guthrie produced these defects by the use of strychnine, caffeine, and nicotine, as had Hertwig, but with concentration far below that of 0.625 sodium chloride. Jenkinson, however, tested out this question of the osmotic pressure of the salt solutions by employing a great variety of isotonic solutions of various salts, such as chlorides, bromides, iodides, nitrates, and sulphates of ammonium, lithium, sodium, potassium, calcium, barium, strontium, and magnesium, and, in addition, solutions of cane sugar, dextrose, urea, and gum arabic. He obtained spina bifida with especial success in his sodium chloride and sodium nitrate solutions. His conclusions are best given in his own words: "There is very little room for doubt, that the malformations in question may be due to some property of a salt other than its osmotic pressure." Bataillon had previously come to the conclusion that malformations were not specific to the means employed. Gurwitsch's belief was that halogens affected the position and development of the blastopore and of the brain, sodium chloride acting upon both, and sodium bromide upon the brain alone, whereas lithium chloride seemed selective on archenteron and blastopore.

It would appear, considering the production of this malformation by the diverse methods outlined above in connection with that detailed by this paper, that we were justified in concluding that in the question of specificity of reaction in the production of spina bifida the weight of argument at present refers the causative forces more particularly to an upset of a specific substance in the egg, rather than a specific action of the agent. It is in the conception of the changes which occur in the chemical composition of the ovum during its differentiation, as previously outlined, that we find support for this statement. It follows that the composition of any particular proanlage or anlage may be such at different stages of its chemical elaboration as to possess a marked affinity for widely varying chemical reagents. The

developmental end-product of the reactions so brought about would be the same, e.g., spina bifida, notwithstanding the wide diversity of character of the chemical reagents employed.

Furthermore, since we cannot deny, we must take into account the possibility of a two-fold manner of production of spina bifida; the one, owing to an upset in the contents of the cells of the unpigmented hemisphere whose yolk is intended for the nutrition or elaboration of the other component, viz., the proanlagen or chemical ferments restricted to the cells of the pigmented hemisphere. For the other, we can assume the possibility of an interference in the function of these proanlagen as the result of the chemical reactions experimentally induced, apart from an upset referable to the composition of the nutritive yolk particles. The author's work, however, points out clearly that in the white hemisphere alone are resident sufficient causes for the production of the malformation, so that, while the possibility of an involvement of the proanlagen exists, the weight of experimental evidence points to the yolk hemisphere as the more vulnerable of the two. Jenkinson observed, for instance, that as the result of chemical action the yolk cells were primarily affected, and Godlewski, employing reduced pressure, came to the same conclusion. The disturbing influences of insufficient aeration and cold, as ascertained by Morgan and others, were noted first in the yolk cells, and to this same region Morgan attributes the causative factors in the results of the centrifuge, while Hertwig drew the same conclusions from studies of overripe eggs.

The production of an area of altered protoplasm, which serves as a mechanical check to the approximation of the lips of the blastopore during the backward progression of the latter, emphasizes very naturally the importance of synchronized tempo in the two processes directly concerned with the elaboration of the neural cord. Under normal conditions the differentiation of the neural anlagen (consequent upon the backward migration of the proanlagen) occurs apparently synchronously with the backward migration of the blastopore and fusion of its dorsal lips. These two processes are approximately coöperative in point of

time, i.e., the anlagen of each half-tube become progressively differentiated in a backward direction at about the time when the half of the dorsal lip in which it is localized meets and fuses with its corresponding fellow of the opposite side. The two processes are not causally dependent upon each other, however, since differentiation takes place in the experimented eggs at about its former rate but now along the equator and not, as usual, parallel to the median plane.

The absence of the proanlagen and anlagen of the egg along the equator in the earliest stages of development of the ovum is sufficiently attested for by the ultra-violet method. Incidentally, it should be remarked that the restriction of these proanlagen at all times to the pigmented hemisphere seems to the author's mind a very significant fact. In this connection, it should be stated that ultimately the yolk mass is wholly drawn into the body of the embryo. Even though by this later process the neural tube halves may be approximated, subsequent fusion does not take place, however, since each half tube has postgenerated into a whole tube.

The conclusions reached by this method of experimentation upon the fertilized ovum, are, therefore; first, that the killing of a small localized area of the yolk hemisphere or of the region of the equator of the frog's egg produces invariably the condition of spina bifida in the embryo; and second, that the neural tube proanlagen, or formative substances, do not lie either in the yolk hemisphere or along the equator of the frog's egg, but are wholly restricted to the pigmented half of the egg. These proanlagen attain their definitive positions by a process of backward migration, the rate of which is synchronous with that of the backward progression of the dorsal lip of the blastopore. The action of the ultra-violet rays in destroying a small localized area of the yolk hemisphere or equator results from mechanical causes in an upset of the synchronism of the two factors, i.e., differentiation of the neural anlagen and approximation of the lips. The former proceeds at its normal tempo, while the latter is retarded. Consequently, the former, always restricted to the pigmented hemi-

sphere, come to lie along the equator and are later carried towards the median plane by the subsequent approximation of the lips, but the half tubes, having already differentiated into whole tubes, do not subsequently fuse.

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ON THE PRESENCE OF INTERSTITIAL CELLS IN THE CHICKEN'S TESTIS

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THREE FIGURES

Since there is a difference of opinion as to whether interstitial cells are present in the testis of the domestic cock, and because of the obvious bearing of the question upon the theory which attributes to these cells an important influence upon secondary sex characters, it has seemed worth while to investigate the matter. To illustrate the difference of opinion I shall abstract briefly two very contradictory reports on the subject.

Alice M. Boring[1] reports observations on testes of roosters from one day to twelve months of age. In the young as well as in the older testes she fails to differentiate any cells of the intertubular tissue from ordinary connective tissue cells. The variation in size, shape and character of the nuclei is attributed to mechanical conditions of pressure. The fat observed in the intertubular tissue was not found inside of the cell bodies, hence it was thought to be brought there by the circulation and deposited. Her conclusion is that there are no interstitial cells present at any time.

In the summary of the work done by J. des Cilleuls,[2] it is stated that the external differentiation of the rooster from the pullet begins to be apparent at about the thirteenth day; and that at this time interstitial cells first make their appearance in the testis. Des Cilleuls says the interstitial cells and cock characters increase *pari passu* and the cock characters are accentuated

[1] Alice M. Boring. The interstitial cells and the supposed internal secretion of the chicken testis. Biological Bulletin, vol. 23, no. 3, August, 1912.

[2] J. des Cilleuls. Interstitial testicular cells and secondary sex-character. Summary in Journal of the Royal Microscopical Society, December, 1912.

while the seminal tubes still remain in an embryonic condition, until after the sixtieth day. The explanation offered is that the internal secretion of the interstitial cells serves as a stimulus for the development of the secondary sex characters.

This report is made after the study of testes from cocks three, five-and-a-half, nine and eighteen months old. The tissue was removed immediately after killing the fowl and fixed in the following solutions: formalin, Zenker's, Bouin's and Ciaccio's fixative:

5% potassium bichromate	20 cc.
formalin	4 cc.
acetic acid	1 cc.

Fix in the above forty-eight hours, then in 3 per cent potassium bichromate one week. Sections were stained chiefly with hematoxylin, and congo red, iron hematoxylin, and Mallory's connective tissue stain. The Ciaccio fixative and Mallory's stain gave the best results for the study of the intertubular tissue, although the other preparations showed up fairly well.

For the study of fat I used frozen sections of tissue fixed in formalin. These were stained with Sudan III and hematoxylin.

Microscopic examination of the sections from the eighteen months testis shows the seminiferous tubules in a state of active spermatogenesis. The intertubular tissue is small in amount and compact, allowing the tubules to lie close together. Where three or more tubules come in juxtaposition small triangular or irregular areas are formed. In most of these areas there is a small blood vessel surrounded by connective tissue which contains both spindle- and oval-shaped nuclei. In other areas there are, in addition to the above structures, typical interstitial cells also, as shown in figure 1. The nuclei are round or oval in outline, rather rich in chromatin and contain an evident nucleolus. The cytoplasm is granular and in certain areas, especially around the nucleus, it is condensed, while near the periphery of the cell it is much less condensed or even vacuolated.

Sections from a five-and-a-half months' testis show the tubules in an inactive state, without spermatozoa, and the intertubular connective tissue slightly greater in amount than in the preceding chicken. The intertubular areas are somewhat larger, but

in other respects the appearances are quite similar to those observed in the cock of eighteen months.

In the sections of the three months' testis, the tubules are much smaller and lined with Sertoli cells, imbedded in which are numerous young sex cells. Relative to the tubules the intertubular spaces are far larger than in any of the older testes. The connective tissue with its spindle-shaped nuclei is readily differentiated from the interstitial cells. The former surrounds the tubules very closely, while the interstitial cells are usually located in the irregular areas formed by three or more tubules

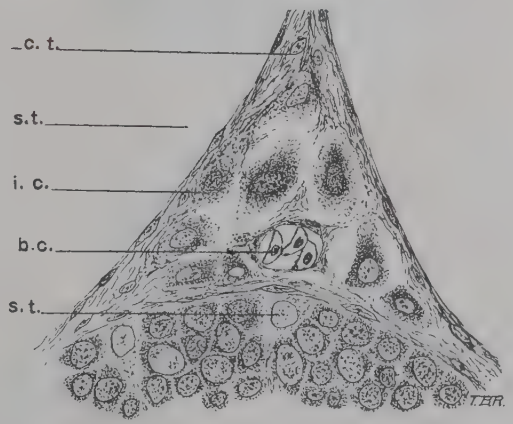
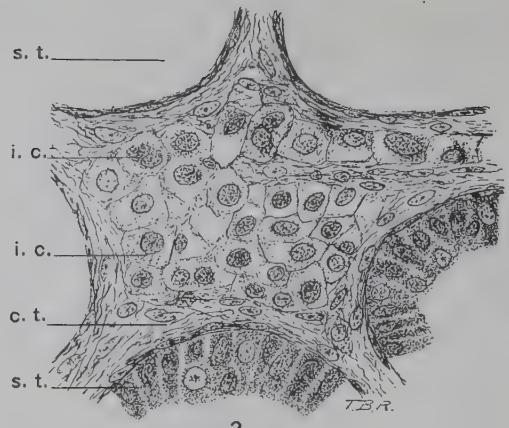


Fig. 1 *C. t.*, connective tissue; *s. t.*, seminiferous tubule; *i. c.*, interstitial cell; *b. c.*, blood cell.

coming close together. In most of these areas there are several interstitial cells; often they form large groups (fig. 2). The cell boundaries are more distinct than in any of the older testes and the cytoplasm is very much more vacuolated. Indeed some of the cell bodies appear almost clear, containing only the nucleus and a small amount of granular cytoplasm.

On examination of the sections stained for fat the interstitial cells in the three months' testis appear to be almost completely filled with fatty material (fig. 3). Thus the vacuolated appearance of the cells in figure 2 is explained. Most of the fat is within the interstitial cells, though there is a good deal free in the intertubular tissue and also a very small amount in the tub-



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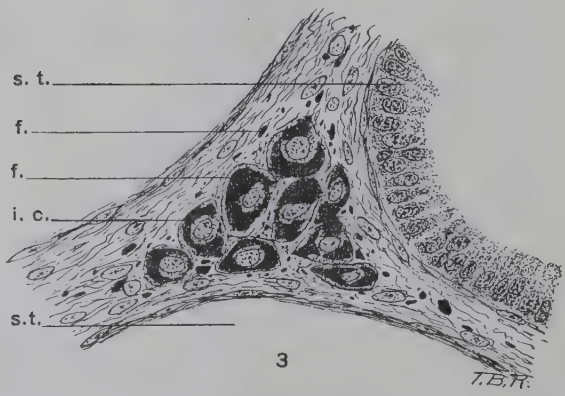

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Fig. 2 *S. t.*, seminiferous tubule; *i. c.*, interstitial cell; *c. t.*, connective tissue.
Fig. 3 *S. t.*, seminiferous tubule; *f.*, fat; *i. c.*, interstitial cell.

ules. In the older testes the fat is very much less in amount and appears as very small particles both in and outside of the interstitial cells. No attempt was made to determine the nature of the fatty material; as it was not rendered insoluble by Ciaccio's fixative, it probably does not consist of phosphatid lipoids to any large extent.

The primary object of this short study was merely to determine the presence or absence of interstitial cells in the testis of the domestic cock; there can be no doubt but that they are present in all the stages examined.

A SIMPLE METHOD OF BRAIN DISSECTION

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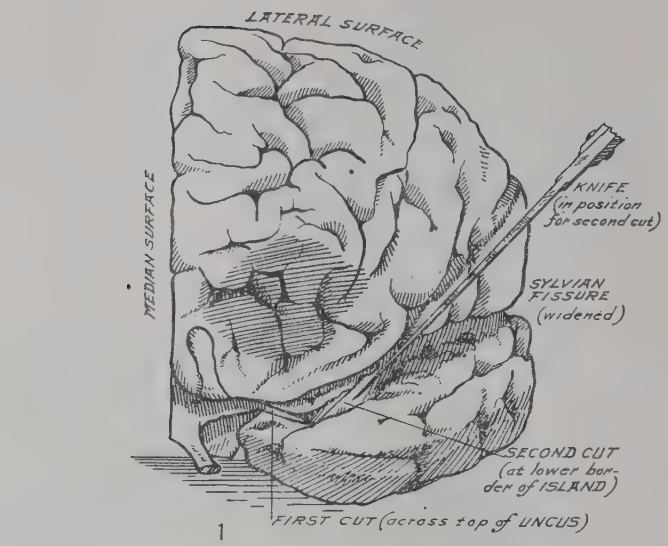
FIVE FIGURES

Every instructor realizes how hard it is to make clear to students the deep or inner structures of the brain. It is difficult to give them a lucid description of even the simpler and more superficial parts, but when it comes to explaining the intricate mechanism, it is an almost hopeless task.

Efforts have been made to disclose the regions, parts, tracts, and nuclear masses by means of a series of cross sections or fiber tract dissections. To all but those well trained in technique and familiar with the general make-up of the brain, these methods are confusing and difficult. Tract dissection necessitates a general understanding of how and where a tract runs, and a series of cross sections presents to a student a mass of labyrinthian vagaries. Most students remember an important structure in a cross section series as it appears in a few well-defined segments, but do not have a clear mental picture or distinct understanding of its extent and relationship. With the following method a student, being guided by a few easily located landmarks, can get the greatest degree of clearness and satisfaction from his work, and have the least amount of cutting and mutilating of tissue.

The procedure is as follows: Using one-half of the brain, clear away all pia mater from the regions to be cut; sylvian fissure, central fissure (Rolandi), post central fissure, superior frontal sulcus, and about the uncus and temporal pole. This is important to make the field of operation perfectly clear and prevent blocking the knife. It is also important to use a long scalpel, the blade of which should be about 7 or 8 cm. long and not more than 1 cm. in width; 0.5 cm. is still better. Place the hemisphere with frontal region upward or toward the student and depress the temporal pole sufficiently clearly to expose the uncus. Now cut across the upper part of this convolution, going from within outward and slightly downward, extending the cut about 2 cm. lateralward and the same backward (fig. 1). Make further depression of the temporal lobe there by widening the sylvian fissure, and cut at nearly right angle to the first incision along the lower border of the island (fig. 1). When this cut is extended 2 or 3 cm. directly backward, the tip of a cavity can be exposed, the anterior extremity of the inferior horn of the lateral ventricle.



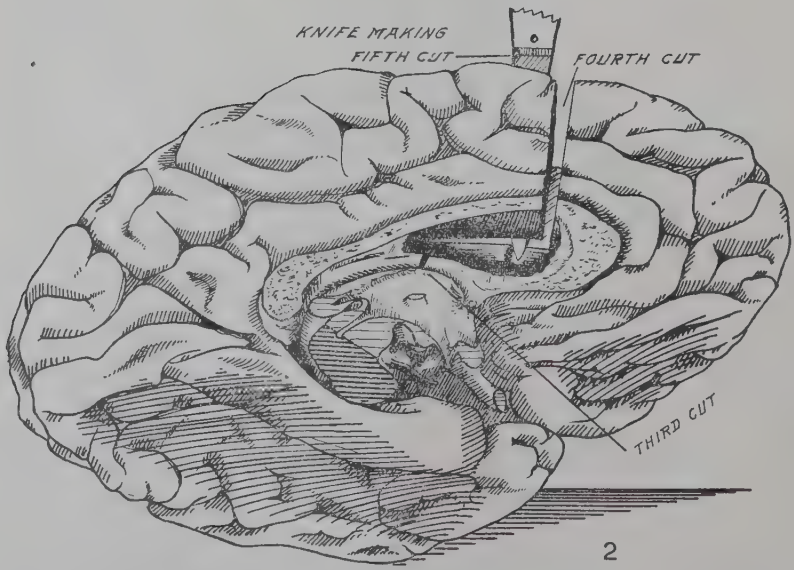
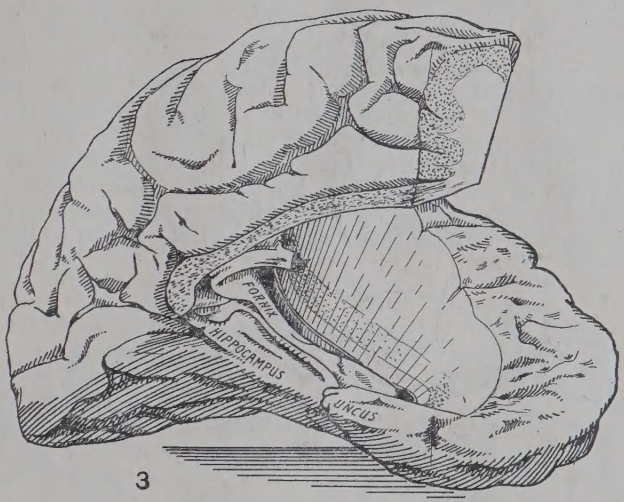


Fig. 1 Showing hemisphere in position, frontal pole forward and temporal pole depressed to make first and second cuts.

Fig. 2 Showing median surface with third and fourth cuts made and knife in position making fifth cut.



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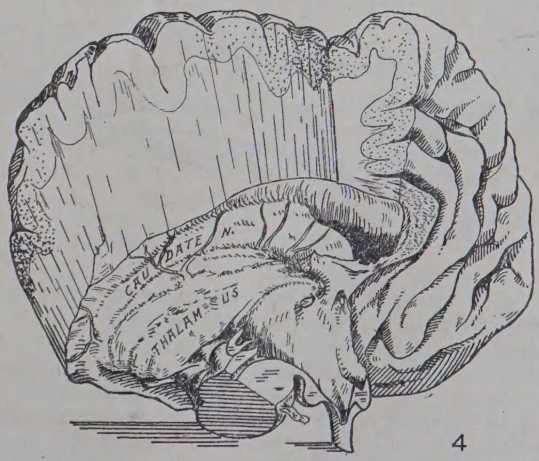

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Fig. 3 Showing 'removable' portion, with hippocampus, fornix, and inferior horn of lateral ventricle.

Fig. 4 Showing 'basal' portion with lateral ventricle and its floor and lateral boundary structure in clear view.

Going now to the median surface of the hemisphere, cut the fornix just back of the foramen of Monroe (fig. 2). Make another cut into the surface of the hemisphere, beginning the incision at a point on the under surface of the corpus callosum just back of where it makes its turn downward, and terminating it on the superior median border a little anterior to the beginning point or directly above the tip of the corpus callosum (genu), thus making an oblique cut. The depth of the incision should extend as far as the superior frontal sulcus on the lateral surface or about 2 cm. from the superior median border, and to the lateral-most extent of the lateral ventricle or just above the caudate nucleus (figs. 2, 5). Now turn the knife at right angles to this plane

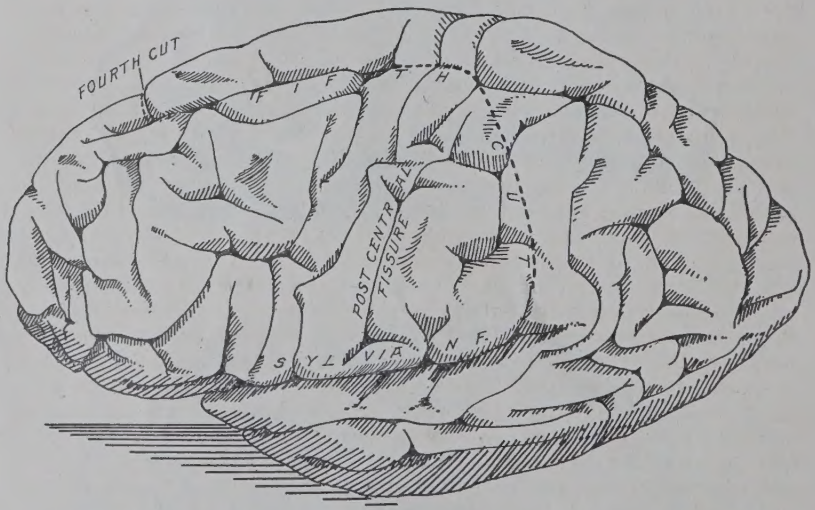

Fig. 5 Showing lateral surface and dotted line marking, on the surface, the course of the shoulder of the knife in making the fifth cut.

and make the following incision with the shoulder of the knife following the superior frontal sulcus or an arbitrary line at about this position, and the point following the lateral-most boundary of the ventricle, cut slowly and gradually backward, all the while elevating the excised portion to give clear view of the field (figs. 2, 5). When the knife comes to the post central fissure, let it swing in a sharp curve downward and backward to the posterior end of the sylvian fissure, reaching this point by following the upper terminal arm of the fissure (fig. 5). While making this curve, the knife should be held with the handle tilted backward so that the point will be a little anterior to the shoulder. This will insure the point cutting through the ventricle at its lateral-most boundary where that cavity turns downward. When

the shoulder comes to the sylvian fissure, lift the handle to about 60° with the horizontal plane, thus forcing the point downward, at the same time turning the sharp edge forward.

Now make a little more bold depression of the temporal lobe through the sylvian fissure, and continue the incision forward along the lower border of the island to meet the original cut at the uncus, all the while holding the knife at about 60° with the horizontal plane.

The incisions have now been completed. With the median surface facing upward, grasp the frontal lobe in the right hand and the occipital lobe in the left hand (this is for the left hemisphere; if the right hemisphere is used, the hands will be reversed), and carefully separate the two portions to about 3 cm. This will stretch out the choroid membrane which can be easily followed almost throughout its entire extent of attachment. When this is carefully studied, the removable portion containing the hippocampal lobe with its fornix can be entirely withdrawn, and a clear view of the lateral ventricle will be had (fig. 3). The complete separation of the two parts will rupture the choroid membrane, but the ragged edges will still give a clear view of the line of its attachment. On the basal portion, in plain view, will be the structures forming the floor and lateral boundary of the lateral ventricle, caudate nucleus, taenia semicircularis, thalamus, etc. The optic tract, geniculate bodies, quadrigeminal bodies, and pes pedunculus can also be easily seen (fig. 4). The two segments can be easily, quickly, and repeatedly separated with no harm whatever to continuity of tissue. When the sections are in place, the cuts are scarcely perceptible; when removed, there is the greatest amount of exposure of hidden structures. A special advantage in this method is that specimens too soft for fiber tract dissection or cross sectioning, or hardened after being mashed or pressed out of shape, can still be used with a good degree of satisfaction when cut as outlined above. There is to be offered this last and most important point, that the removable segment comes off from the basal portion in approximately the same course the hemisphere pursued in its early stages of development. Following this course of development as displayed by such a method of removing part of the hemisphere, it is much easier for the student to see how the velum interpositum was at one time a part of the wall, the roof portion of the forebrain, of the neural tube, and its presence in the fully developed specimen, attached to the sharp edge of the fornix on the one side and the taenia semicircularis and thalamus on the other, makes a closed cavity of the lateral ventricle and its horn.

This method is not to be used for complete work; the nuclear masses and fiber tracts demand deeper dissection. But using the method on one hemisphere and cross section on the other, the student will have far greater and more gratifying results, and will have good material, easily kept, for future reference.

Finally, I wish to express my appreciation to Dr. Bremer and Mr. Miller for their kindness in reviewing this paper and making valuable suggestions.

BOOKS RECEIVED

The receipt of publications that may be sent to any of the five biological journals published by The Wistar Institute will be acknowledged under this heading. Short reviews of books that are of special interest to a large number of biologists will be published in this journal from time to time.

THE ANATOMY OF THE DOMESTIC ANIMALS. By Septimus Sisson, S.B., V.S., Professor of Comparative Anatomy, Ohio State University, College of Veterinary Medicine. Second edition entirely reset. Octavo of 930 pages, 724 illustrations. Philadelphia and London: W. B. Saunders Company, 1914. Cloth, $7.00 net; Half Morocco, $8.50 net.

Preface to first edition. The lack of a modern and well-illustrated book on the structure of the principal domestic animals has been acutely felt for a long time by teachers, students, and practitioners of veterinary medicine. The work here offered is the expression of a desire to close this gap in our literature.

The study of frozen sections and of material which has been hardened by intravascular injection of formalin has profoundly modified our views concerning the natural shape of many of the viscera and has rendered possible much greater precision in topographic statements. The experience of the author during the last ten years, in which almost all of the material used for dissection and for frozen sections in the anatomical laboratory of this University has been hardened with formalin, has demonstrated that many of the current descriptions of the organs in animals contain the same sort of errors as those which prevailed in regard to similar structures in man previous to theadoption of modern methods of preparation.

While the method of treatment of the subject is essentially systematic, topography is not by any means neglected either in text or illustrations; it is hoped that this will render the book of value to the student in his clinical courses and to the practitioner. Embryological and histological data have been almost entirely excluded, since it was desired to offer a text-book of convenient size for the student and a work of ready reference for the practitioner. * * *

Preface to second edition. This book supersedes the author's Text-book of Veterinary Anatomy. A comparison of the two will show the new title to be justified by the extent and character of the changes which have been made.

Continued observations of well-hardened material and frozen sections have led to a considerable number of modifications of statement. It is scarcely necessary to say that the recent literature, so far as available, has been utilized.

Many changes in nomenclature have been made. Most of the synonyms have been dropped or relegated to foot-notes. Exceedingly few new names have been introduced. Nearly all eponyms have been eliminated, on the ground that they are not designative and are usually incorrect historically. The changes made in this respect are in conformity with the report of the Committee on Revision of Anatomical Nomenclature which was adopted by the American Veterinary Medical Association two years ago. Progress in the direction of a simplified and uniform nomenclature is much impeded by the archaic terminology which persists to a large extent in clinical literature and instruction. * * *

SEPTIMUS SISSON.

The Ohio State University, Columbus, Ohio,
September, 1914.